

Ecdysone-dependent proteolysis of an apical surface glycoprotein may play a role in imaginal disc morphogenesis in *Drosophila*

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Summary

An apical surface glycoprotein, designated gp125 for its apparent molecular weight of 125 000, appears in Ca²⁺-free, ionic detergent extracts of imaginal discs of *Drosophila melanogaster* in response to the steroid hormone, 20-hydroxyecdysone (20-HE). Gp125 is not synthesized in response to 20-HE, but results from modification of an existing macromolecule. Treatment of discs or larval epidermis with serine protease (e.g., trypsin) results in hormone-independent production of gp125. Antiserum raised to electrophoretically purified gp125 recognizes, in addition to gp125, two closely related glycoproteins with higher apparent molecular weights, gp200 and

gp180. This family of glycoproteins is localized at the apical surface of imaginal disc cells and of the epidermal epithelium in embryos, larvae and prepupae. Ca²⁺ affects both the solubility and the proteolytic products of this family of glycoproteins. We discuss the possibility that gp125 is generated through the action of a hormonally controlled serine protease in a process that is necessary for disc morphogenesis.

Key words: imaginal disc morphogenesis, *Drosophila*, cell surface glycoprotein, proteolysis, ecdysone.

Introduction

The precise molecular means by which cells become organized into complex spatial patterns during development are unknown. However, modulations in cell-surface molecules are likely to be involved in morphogenetic cell movements. Specific examples are provided by the cell–cell adhesion molecules such as the cadherins (Takeichi, 1988) and CAMs (Edelman, 1986), as well as cell–substrate adhesion molecules such as the integrins (Hynes, 1987; Bogaert *et al.* 1987; Leptin *et al.* 1989). In this paper, we characterize a surface glycoprotein family that we propose may be involved in imaginal disc morphogenesis during *Drosophila* metamorphosis.

Imaginal discs of *Drosophila* are epithelial organs that arise as invaginations of the embryonic epidermis, increase in size by cell division during larval life and, during metamorphosis, form most of the external structures of the adult (Nöthiger, 1972). At pupariation, discs undergo morphogenesis in response to the steroid hormone, 20-hydroxyecdysone (20-HE) (Borst *et al.* 1974; Fristrom and Fristrom, 1975). Morphogenesis appears to be dependent on 20-HE-induced *de novo* RNA and protein synthesis (Fristrom *et al.* 1973). Imaginal discs, mass isolated from late third instar larvae and incubated with 20-HE, undergo morphogenesis *in vitro*, providing a convenient model to study the

molecular and cellular mechanisms that underlie epithelial morphogenesis.

Each larval disc is an epithelial sac that sits within the body cavity and is typically connected to the larval epidermis by a short stalk. One side of the disc is a folded columnar epithelium, the disc proper; the opposite side is a flattened epithelium, the peripodial epithelium. The disc epithelium encloses a lumen which opens, via the stalk, to the extracellular space beneath the larval cuticle. The apical epithelial surface faces the lumen of the disc and is continuous with the apical surface of the larval epithelium. The basal surface faces the larval hemocoel. Morphogenesis of a disc typically involves (1) elongation of the disc proper to form an appendage within the lumen of the disc and, (2) eversion of the elongated appendage through the stalk to the exterior of the animal. Elongation and eversion, together called evagination, result in the formation of the basic shape of an appendage (e.g., an antenna, wing or leg). Evagination results from cell shape changes (Milner *et al.* 1984; Condic *et al.* 1990) and cell rearrangements (Fristrom and Fristrom, 1975; Fristrom, 1976). These processes presumably require changes in cell–cell adhesions and the transmission of signals from the cell surface to specific cytoskeletal elements.

The syntheses of new disc cell surface proteins in response to 20-HE have been described (Rickoll and Fristrom, 1983 and Woods *et al.* 1987). In addition,

three hormone-dependent genes (IMP-E1, -E2 and -E3) are transcribed in disc cells at the onset of evagination (Natzle *et al.* 1988; Paine-Saunders *et al.* 1990; Moore *et al.* 1990). However, 20-HE-dependent modifications of cell surface components associated with evagination may arise by mechanisms other than *de novo* synthesis. Serine proteases are known to accelerate *in vitro* evagination of *Drosophila* (Poody and Schneiderman, 1971; Fekete *et al.* 1975), and of *Manduca* discs (Nardi *et al.* 1987). Evagination of *Drosophila* discs occurs in approximately 16 h both *in vivo* and *in vitro*, with morphological differences first detected 4–6 h after an initial essential exposure to 20-HE. After the 4–6 h exposure of discs to 20-HE, evagination can be accelerated by exposure of discs *in vitro* to 0.1 % trypsin. These previous observations did not determine whether protease-accelerated evagination resulted from modulation of specific key substrates or was the consequence of a general loss of adhesion from random digestion.

Recent findings of the 20-HE-dependent appearance of extracellular proteases in *Drosophila* discs suggest that proteolysis itself may be an inherent part of the mechanism of evagination (Pino-Heiss and Schubiger, 1989). The data presented here support this view by identifying a putative key product of a trypsin-like serine protease at the disc cell surface. We describe an apical surface glycoprotein (gp125), soluble in ionic detergents in the absence of Ca^{2+} , which appears to be generated from an existing macromolecule during the course of 20-HE-induced morphogenesis and which can be generated independently of 20-HE by trypsin treatment of discs.

Materials and methods

Isolation and culture of imaginal discs

An Oregon R strain maintained in our laboratory since 1964 and reselected using pair matings for increased fecundity in 1986 was used as a source of imaginal discs. Discs were mass-isolated (Eugene *et al.* 1979) and cultured ($3000\text{--}5000\text{ ml}^{-1}$) at 25°C in 25 ml Robb's medium (Robb, 1969) supplemented with 1 % bovine serum albumin (BSA). 20-HE was continuously present at a concentration ($1\ \mu\text{g ml}^{-1}$) that promotes morphogenesis in the absence of cuticle formation (Doctor *et al.* 1985; Fristrom and Liebrich, 1986). After 12 h of culture under these conditions, evagination was approximately 3/4 complete, i.e. leg discs had an evagination index of 6–8 out of 10 (10 represents full evagination; Chihara *et al.* 1972).

Radiolabeled amino acids ($[^3\text{H}]$ amino acid mixture, ICN; or $[^{35}\text{S}]$ methionine, Amersham) were used at $10\ \mu\text{Ci ml}^{-1}$ of Robb's culture media lacking the corresponding amino acid(s). Discs were incubated with radiolabeled precursors for 4 h at various times as indicated.

Preparation and partial purification of gp125

Prior to sequential detergent extraction, discs were washed in one of two buffers (TE and TC). The standard buffer, TE, contained 1 mM EDTA in 10 mM Tris-HCl, 1 mM phenylmethylsulfonyl fluoride (PMSF), pH 7.6. In experiments where Ca^{2+} was present, TC buffer was used (10 mM Tris-HCl, 1 mM

CaCl_2 , 1 mM PMSF, pH 7.6). Except where noted, discs were homogenized and the crude membrane pellet was extracted with detergents. Membranes were extracted for 24 h at 4°C , first with 1 % Triton-X 100 in TE or TC and subsequently with 1 % sodium deoxycholate (DOC) in TE or in 10 mM Tris, pH 7.6 (The Ca^{2+} salt of DOC is insoluble). The insoluble material was then extracted for 2 min, 100°C with 2 % sodium dodecyl sulfate (SDS). The SDS-insoluble pellet was finally extracted at 100°C for 2 min in 2 % SDS containing 5 % mercaptoethanol (Laemmli, 1970). Protein concentrations in detergent extracts were determined by a modification of Lowry's method (Dulley and Grieve, 1975).

For partial purification of gp125 by HPLC, crude membranes from discs incubated with 20-HE for 12 h were sequentially extracted with Triton-X 100 and DOC. The remaining pellet was extracted with 50 % acetonitrile in water containing 0.1 % trifluoroacetic acid for 48 h, 22°C , and then for 48 h, 4°C . The organic solvent soluble fraction was subjected to size exclusion HPLC on a $0.7\times 60\text{ cm}$ TSK 5000 PW column eluted with the extraction solvent at 0.6 ml min^{-1} .

Immunization

Antiserum against gp125 was produced in rabbits using the protocol of Knudsen (1985). In brief, the HPLC fraction containing gp125 was subjected to SDS-PAGE and subsequently blotted onto nitrocellulose. The region containing gp125 was cut from nitrocellulose blots and the protein was dissolved in 100 % dimethylsulfoxide. Rabbits were immunized, using standard techniques, with a total of approximately 1 mg of gp125.

Characterization of proteins on western blots

SDS-PAGE used 7.5 % polyacrylamide and the conditions of Laemmli (1970). Two-dimensional gel electrophoresis used the methods of O'Farrell (1975). Standard proteins (Amersham) were used as molecular weight markers. Proteins were detected in gels with Coomassie Blue (Fairbanks *et al.* 1971), on nitrocellulose with india ink or colloidal gold (Sigma).

Immunochemical detection used horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin as the secondary antibody and 4-chloro-1-naphthol as substrate. Nitrocellulose blots were blocked with 5 % non-fat milk in Tris-buffered saline (10 mM Tris-HCl, 154 mM NaCl, pH 7.4). Wash buffers contained 0.1 % NP-40 in Tris-buffered saline or phosphate-buffered saline (PBS, 10 mM phosphate, 154 mM NaCl, pH 7.2). Anti-gp125 antiserum was used at a dilution of 1:2500.

Carbohydrate detection used peroxidase hydrazide (Gershoni *et al.* 1985) or biotinylated lectins (Vector laboratories) and avidin-conjugated peroxidase.

Protease treatment and analysis of peptides

Tissue fractions prepared as described above were incubated with 0.1 % trypsin (treated with TPCK to remove chymotrypsin activity) or with 0.1 % chymotrypsin (Cooper Biomedical), in TE or TC for 15 min, 22°C . The reaction was stopped by the addition of 1 mM PMSF (Sigma). Subsequent washes and extractions contained PMSF. Collagenase (affinity purified; gift of Lisa Fessler) was used in a buffer containing 50 mM NaCl, 10 mM CaCl_2 , 20 mM Tricine-HCl, pH 7.4.

The method of Cleveland *et al.* (1977) was used to compare proteolytic cleavage products in 14 % SDS-PAGE. Gel strips containing gp200 or gp125 were placed in wells and overlaid with V-8 protease ($25\ \mu\text{l}$, 0.5 mg ml^{-1} , 720 U mg^{-1} , Sigma) or chymotrypsin ($25\ \mu\text{l}$, 2 mg ml^{-1} , 54 U mg^{-1} , Sigma). The gel strips were cut from SDS gels after the regions containing gp125 and gp200 were located by overlaying the gel on a

stained nitrocellulose diffusion blot. The unfixed gel was frozen at -70°C and stored at -20°C while the nitrocellulose copy was processed.

Fluorescence microscopy

Immunofluorescent localization of gp125 was performed on $8\ \mu\text{m}$ frozen sections of late 3rd instar larvae and 4 h prepupae. Sections were fixed in 4% formaldehyde in PBS, blocked with 1% BSA (1 h), and then incubated for 2 h with antibody diluted 1:500. Primary antibody binding was detected with biotinylated goat anti-rabbit IgG antibody followed by fluorescent avidin (Vector). Whole-mount preparations of 2–5 h embryos (permeabilized according to Patel *et al.* 1989) and 2 h prepupal discs were fixed as above and incubated overnight in primary antiserum diluted 1:1000 in PBS with 0.5% Triton X100. Detection was with goat anti-rabbit IgG conjugated directly with FITC (Cappel) observed with a scanning confocal microscope (Biorad MRC500).

Results

Gp125 is a 20-HE-dependent glycoprotein

The one-dimensional electrophoretic patterns of proteins fractionated by sequential detergent extraction of discs cultured *in vitro* with or without 20-HE ($1\ \mu\text{g}\ \text{ml}^{-1}$) are shown in Fig. 1. Only one Coomassie Blue-positive band (in the SDS-soluble fraction) is hormone-dependent. This has been designated gp125 for its apparent molecular weight of 125 000. Gp125 can also be extracted from hormone-treated discs with other strong denaturants, e.g. 7 M urea, 6 M guanidine hydrochloride, and 50% aqueous acetonitrile containing 0.1% trifluoroacetic acid (data not shown). The glycoprotein nature of gp125 was demonstrated by staining with reagents that react with sugar, periodic acid/Schiff's reagent (PAS) in gels (not shown) and peroxidase hydrazide on nitrocellulose blots (Fig. 2). Gp125 is also reactive with lectins, including Concanavalin A (lane 3), UEA I (lane 4), and DBA (lane 6) suggesting that it contains mannose, galactose and fucose (Fig. 2). Other lectins [wheat germ, soybean, peanut, and RCA (lanes 2, 5, 7 and 8)] were not found to bind to gp125.

GP125 is related to two other glycoproteins, gp200 and gp180

The specificity of polyclonal antiserum, made to HPLC and electrophoretically purified gp125, was characterized by immunoblotting (Figs 2, 4 and 6). In addition to gp125, the antiserum has strong immunological cross reactivity with two other glycoproteins having higher apparent molecular weights, gp200 and gp180 (Fig. 2). Like gp125, gp200 and 180 are peroxidase hydrazide-positive and are stained by Con A and DBA (gp200 only). Unlike gp125, they are RCA-positive. The relatedness of gp125 and gp200 was compared by one-dimensional proteolytic cleavage patterns (Cleveland *et al.* 1977; gp180 was not tested because we could not recover it from polyacrylamide gels). The peptides produced by proteolysis of gp200 and gp125 were highly similar to one another using two different proteases (Fig. 3). We conclude from these results that gp200 and gp125 share a common primary structure.

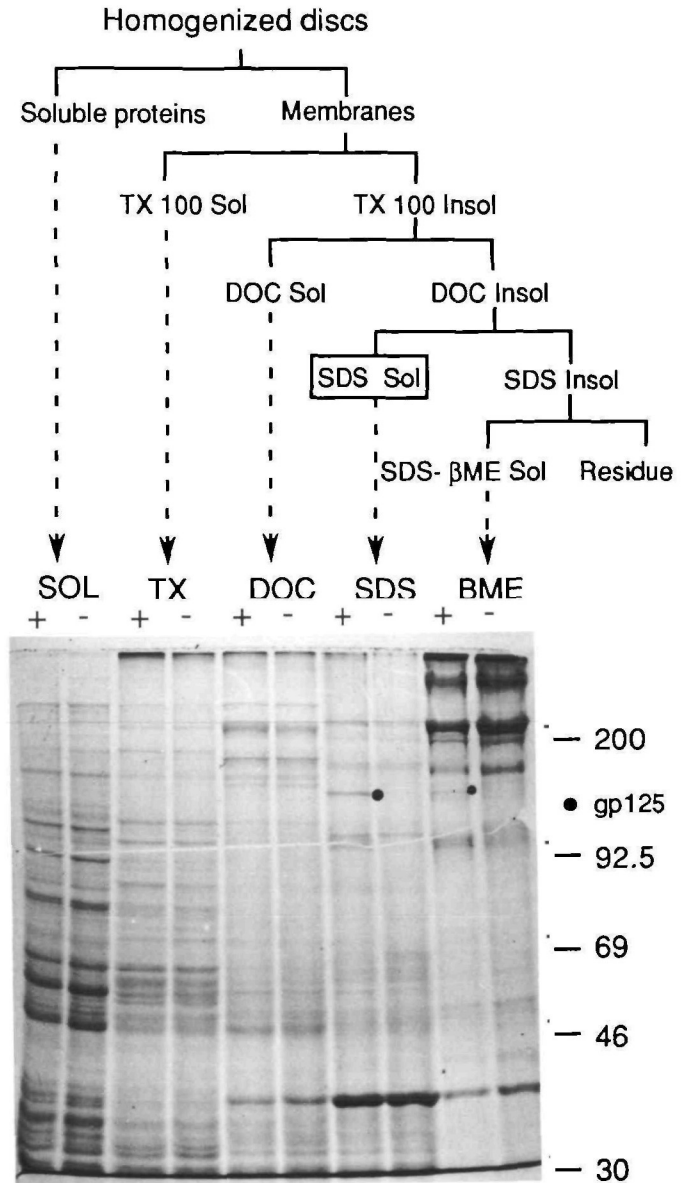


Fig. 1. Disc proteins are fractionated by their differential solubility in detergents. Discs were incubated with (+) and without (–) 20-HE for 12 h prior to sequential extraction with detergents as shown in the flow chart. Samples ($75\ \mu\text{g}$ protein/lane) were subjected to 7.5% SDS-PAGE and detected by Coomassie Blue staining. SOL, soluble protein (approx. 50% of the total protein); TX, Triton X100-soluble (32% of total protein); DOC, DOC-soluble (12%); SDS, SDS-soluble protein (5%); BME, protein ($>1\%$) soluble in Laemmli sample buffer (2% SDS, 5% beta-mercaptoethanol, 2 min at 100°C). Dot indicates position of gp125. Standard molecular weights (as marked $\times 10^3$): 200 000, myosin; 92 500, phosphorylase B; 69 000, bovine serum albumin; 45 000, ovalbumin; 30 000, carbonic anhydrase.

Although gp200 and possibly gp180 appear to be higher molecular weight forms of gp125, they differ from gp125 in two respects. First, and most important, the presence of gp200 and 180 is not 20-HE-dependent; both are found in substantial amounts in control discs

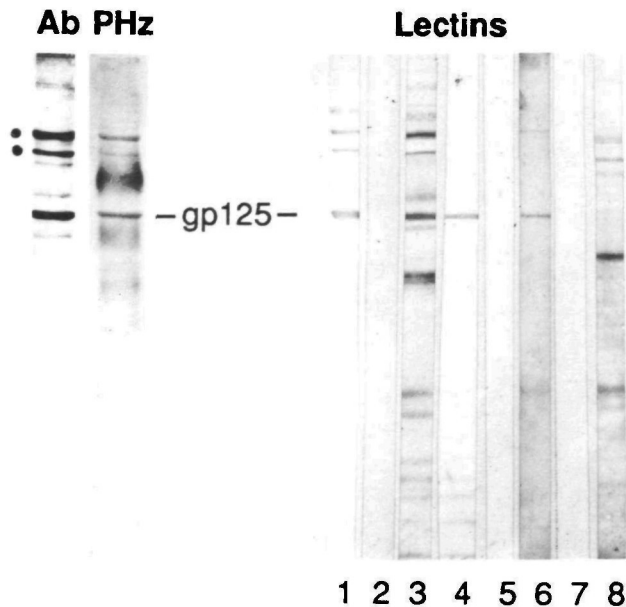


Fig. 2. Gp125 is a lectin reactive glycoprotein. SDS-soluble proteins from sequential detergent extraction of discs incubated 12 h with 20 HE were subjected to SDS-PAGE, transferred to nitrocellulose, and stained as indicated. Ab, anti-gp125 antiserum detection of gp125 and related antigens; PHz, peroxidase-hydrazide detection of carbohydrate after periodic acid oxidation. Dots indicated positions of gp200 and gp180. Lectins: 1, Reference lane stained with anti-gp125; 2, Wheat germ agglutinin; 3, Concanavalin A; 4, UEA I; 5, Soybean agglutinin; 6, DBA; 7, Peanut agglutinin; 8, RCA.

(Fig. 4). Indeed, as we will demonstrate, these higher molecular weight forms are present in epithelial tissues throughout most of development. Second, the solubility of gp200 and 180 differs from that of gp125. They are always present in the SDS-mercaptoethanol-soluble fraction and are either not extracted (Fig. 4B) or only partly extracted with 2% SDS (Fig. 4A).

It is apparent from Fig. 4 and subsequent figures that gp125 is often present at low levels in control lanes. This may be an artifact that could stem from two sources. First, discs were mass-isolated from late third instar mass-reared larvae. Typically, some larvae are close to pupariation and their discs have been exposed to 20-HE *in vivo*. Second, because gp125 can be produced by proteolysis *in vitro* (Fig. 6), it can be produced inadvertently unless great care is taken to minimize proteolytic activity during membrane isolation (see Methods). Even if the low levels of gp125 in control discs are not artifactual, incubation with 20-HE significantly increases the amount of gp125 present (Figs 4 and 8).

Gp125 is generated by a hormone-dependent modification of an existing molecule

In order to determine the origin of gp125 in the SDS-soluble fraction of hormone-treated discs, we first examined the possibility that gp125 was synthesized *de novo* in response to 20-HE. Using two-dimensional gel electrophoresis, no radiolabel from either [³⁵S]methionine or [³H]amino acids (a mixture of 15, excluding C,

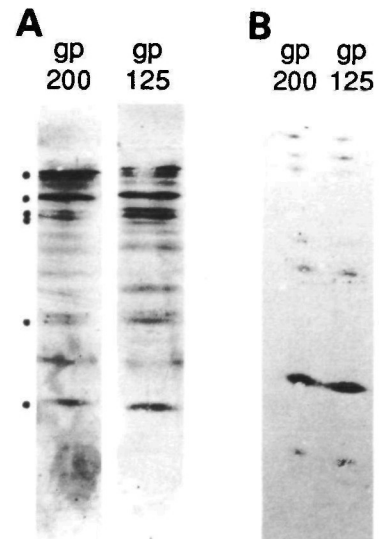


Fig. 3. Two separate comparisons of the peptides made by proteolytic cleavage of gp125 and gp200 show that they are closely related. The regions containing gp125 and gp200 were cut from 7.5% SDS gels and were subjected to SDS-PAGE in 14% gels after digestion with V8 protease (A) or chymotrypsin (B) as described in Methods. (A) Nitrocellulose blot of peptides generated with V8 protease and double stained with colloidal gold and with anti-gp125 antiserum (dots indicate bands detected with both stains). (B) Peptides generated with chymotrypsin and detected with anti-gp125 antiserum.

M, Q, N, and W) became incorporated into gp125 at anytime during hormone treatment (Fig. 5). Incubations with radiolabeled precursors were from 0–4 h, 4–8 h, and 8–12 h of *in vitro* culture with 20-HE (data shown for 8–12 h), or were for 2 h before hormone addition. Immunoprecipitation with anti-gp125 also confirmed that gp125 and its related antigens do not incorporate label over the course of *in vitro* culture (data not shown). We conclude that gp125 arises by modification of an existing molecule rather than by *de novo* synthesis.

Trypsin generates gp125

When freshly isolated discs (not exposed to 20-HE) are homogenized and treated briefly with 0.1% trypsin, the amount of gp125 in the SDS-soluble extracts increases dramatically compared to untreated controls (Fig. 6A). This suggested that gp125 might be a proteolytic cleavage product of gp200 (or gp180). We attempted to demonstrate this relationship by converting electrophoretically purified gp200 to gp125 by trypsin digestion *in vitro*. A number of low molecular weight peptides were generated but not gp125. We failed, therefore, to support a precursor/product relationship between these two glycoproteins. This does not rule out the possibility that such a relationship exists. More sites for proteolysis may be available on the denatured molecule than on the native one resulting in an array of smaller peptides.

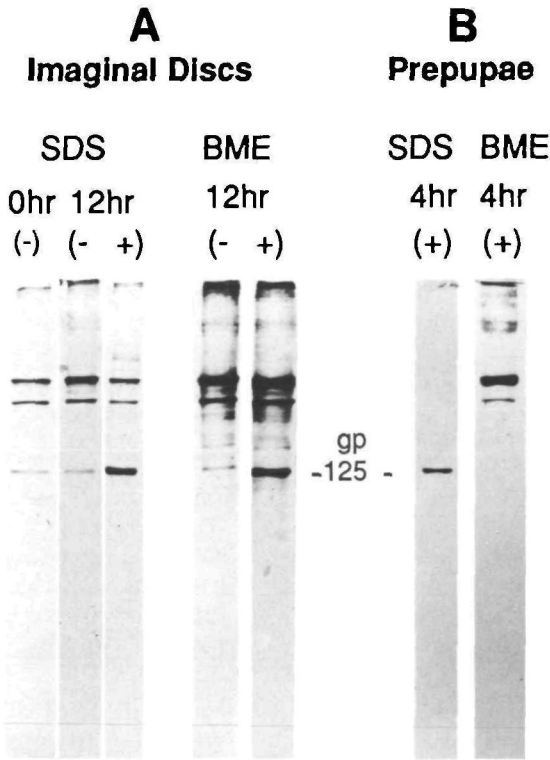


Fig. 4. Three related glycoproteins, gp125, 180 and 200 are detected by anti-gp125 antiserum on Western blots of SDS-soluble proteins (SDS) and SDS-mercaptoethanol-soluble proteins (BME) isolated from imaginal discs and prepupae. Imaginal discs were unincubated (0 h) or incubated with (+) and without (-) $1 \mu\text{g ml}^{-1}$ 20-HE. Note that gp125 is only abundant in hormone-treated discs whereas gp180 and 200 are equally abundant in the presence and absence of hormone. Prepupae (4 h after puparium formation) have been exposed to 20-HE *in vivo*: Note the differential solubility of these three antigens in prepupae compared with discs.

Gp200, and 180 are present in the larval epidermal epithelium

Immunostaining of sections of whole larvae and pupae shows reactivity of the anti-gp125 antiserum with epidermal epithelium (Fig. 7A) as well as with disc epithelium (Fig. 7B and C) but not with any other tissues. Staining with the antiserum was first detected in localized regions of the apical epidermal surface in stage 10–11 embryos (at the end of germ band extension, Fig. 7D). By stage 12, staining was continuous over the apical epidermal surface.

In homogenized larvae of all stages (Fig. 6B), gp125 is produced by trypsin treatment and gp200 and 180 are present in untreated controls. Discs constitute only a small amount of the total tissue in larvae at least until the mid third instar, so we assume that most of the gp125 family of glycoproteins observed in this experiment were derived from larval epidermis.

We then asked whether gp125 could be generated in the epidermis in response to 20-HE as it is in discs. We compared dissected integuments from mid-third instar larvae (i.e. before and after the metamorphic rise of 20-

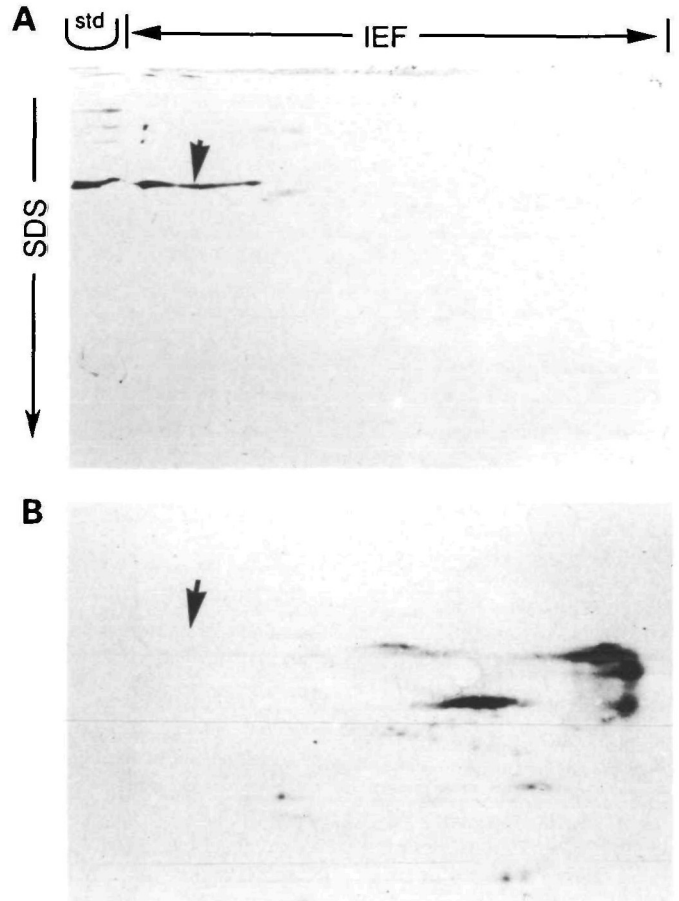


Fig. 5. Antibody staining of radiolabeled SDS-soluble proteins shows that gp125 is not synthesized *de novo* during morphogenesis. Discs were incubated with $10 \mu\text{Ci ml}^{-1}$ [^{35}S]methionine for 4 h starting 8 h after addition of 20-HE to discs in Robb's medium lacking methionine. Radiolabeled, SDS-soluble proteins were subjected to two-dimensional electrophoresis and transferred to nitrocellulose. (A) Gp125 was detected with anti-gp125 antiserum. (B) Autoradiogram of the same gel. First dimension: isoelectric focusing, pH 4–8, acid end to right. Second dimension: 7.5 % SDS-PAGE. Well on left: SDS-soluble proteins from discs incubated 12 h with 20-HE.

HE titer *in vivo*) and 4 h prepupae of wild-type and two discless mutants (*B2(1)102.2* and *B3(1)1189.2*) isolated by Paine-Saunders *et al.* (1990). In all cases, prepupal integuments contained gp125, whereas larval integuments contained gp200 and 180 but little or no gp125 (not shown).

Gp200, 180 and 125 are located at the apical epithelial surface

Immunochemical staining of whole mounts and frozen tissue sections showed that anti-gp125 antiserum specifically bound only to the apical surface of disc epithelia as well as to the apical surface of embryonic, larval and prepupal epidermal epithelia (Fig. 7). (Later stages of development were not examined.) Because significant signal is observed only at the apical epithelial surface, we conclude all three of these glycoproteins are

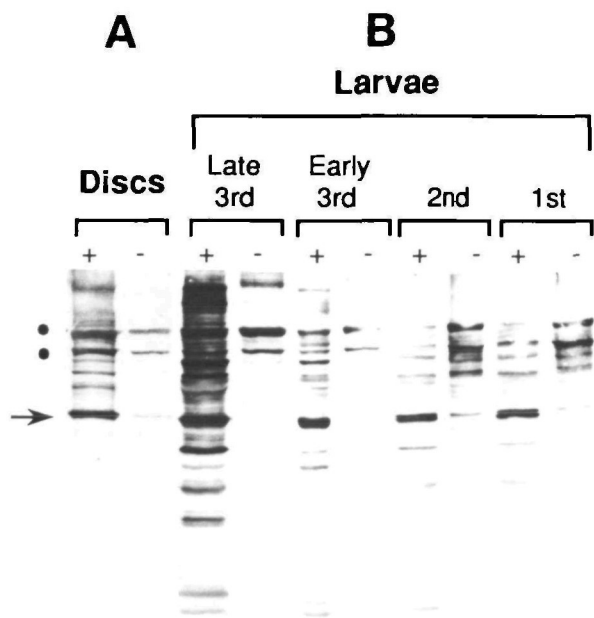


Fig. 6. Addition of protease generates gp125 in (A) freshly mass-isolated third instar discs and (B) larvae at different stages of development. Tissues were homogenized and incubated for 20 min with 0.1% trypsin in TE buffer (+) or in TE buffer without trypsin (-). Gp125 and related antigens were detected by anti-gp125 on nitrocellulose after SDS-PAGE of the SDS-soluble fractions obtained by sequential detergent extraction of samples. 20 μg protein/lane except for late third instar with 32 μg /lane. Note that gp180 and gp200 (dots) are present throughout whereas gp125 (arrow) is only abundant after protease treatment.

apically located. In late third instar larvae, gp125 is not present in significant amounts, so the apical signal is presumably due to gp200 and 180 or to an insoluble precursor. In an intact disc, the apical surface faces the lumen and is less accessible than the basal surface to exogenous proteases. Trypsin was substantially less effective in generating gp125 from intact discs than from discs disrupted by very brief homogenization (that generates cell clumps with the apical surface exposed; data not shown). This result is consistent with the apical location of gp125 and its precursor(s).

The appearance of gp125 correlates with the onset of imaginal disc morphogenesis

When the *in vitro* time course of the hormone-dependent appearance of gp125 in discs was investigated, increased amounts of gp125 were detected prior to any visible morphogenesis, i.e. within 2 h by antibody detection on nitrocellulose (Fig. 8). Gp125 continued to be present for at least 12 h (Fig. 8). The 20-HE concentrations ($1 \mu\text{g ml}^{-1}$) routinely used in these experiments induce disc morphogenesis but substantially inhibit cuticle formation. At a hormone concentration

($0.1 \mu\text{g ml}^{-1}$), which supports both cuticle synthesis and evagination, gp125 was generated in comparable amounts. A concentration of 20-HE ($0.001 \mu\text{g ml}^{-1}$) which did not induce evagination, did not result in the appearance of gp125 (data not shown).

Discs incubated *in vitro* with 20-HE and the serine protease inhibitor, aprotinin (1 mg ml^{-1}) do not accumulate gp125 (not shown), consistent with the hypothesis that the hormonally induced appearance of gp125 occurs via proteolytic cleavage. Aprotinin at this concentration partially inhibits evagination of dissected discs (Pino-Heiss and Schubiger, 1989). In our hands, using mass-isolated discs, aprotinin completely inhibited evagination but was also toxic after 12 h of incubation.

Treatment of discs with chymotrypsin generates one immunoreactive protein that comigrates with gp125 on SDS gels (Fig. 9A). This is somewhat surprising in view of the different cleavage site specificities of trypsin and chymotrypsin. However, we do not know if the two comigrating bands generated by these two proteases are identical; their similar sizes may result from a fortuitous arrangement of accessible cleavage sites on the precursor molecule. Alternatively, both these enzymes might work indirectly by activating an endogenous protease. The important point is that both trypsin and chymotrypsin markedly accelerate disc evagination and generate gp125. In contrast, highly purified collagenase (gift of Lisa Fessler), which digests disc basal lamina (D. Fristrom, unpublished observations), does not produce gp125 (Fig. 9A) nor does it affect the rate of disc morphogenesis.

Ca²⁺ affects the solubility and protease sensitivity of gp125

Ionic detergent extractions of disc membranes routinely included EDTA in the buffers. When Ca²⁺ was present (EDTA absent), gp125 was absent from the SDS-soluble fraction of hormone-treated discs (Fig. 9B). Gp125 could subsequently be extracted in detergent after the removal of Ca²⁺ (data not shown). Furthermore, when Ca²⁺ was present during incubations with trypsin, a smaller fragment (apparent molecular weight of 51 000) as well as gp125 appeared in the SDS-soluble fraction after subsequent Ca²⁺-free extraction (Fig. 9A). This molecule was not found in chymotryptic digestions or when trypsin was used without Ca²⁺ present.

Discussion

Gp125 is a glycoprotein that is formed at the apical surface of imaginal discs and epidermal epithelia in response to the steroid hormone 20-HE. It is not synthesized *de novo* in response to hormone but appears to be generated by proteolysis of a precursor. We have identified two other glycoproteins, gp200 and gp180, that are related, both immunologically and, in the case of gp200, in primary sequence to gp125 (based on one-dimensional 'fingerprints'). All three proteins

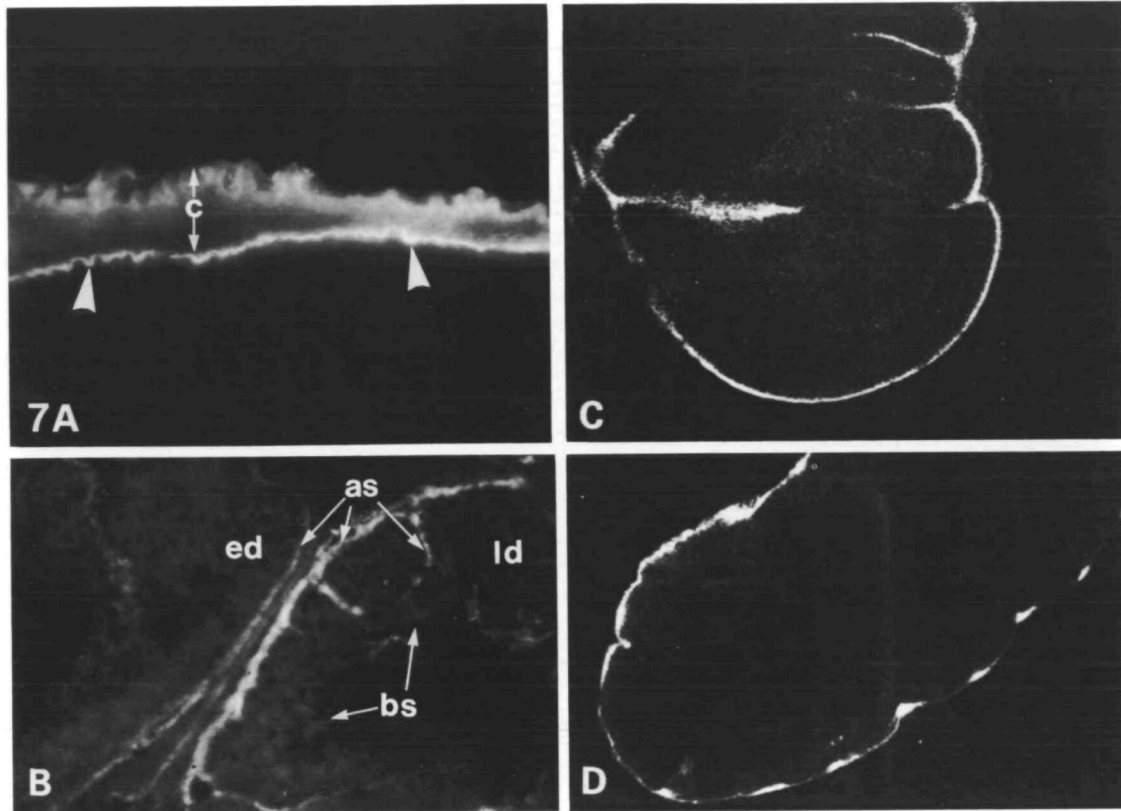


Fig. 7. Immunofluorescent localization of the gp125 family of glycoproteins. (A,B) Fluorescent micrographs of a frozen $8\ \mu\text{m}$ section of a late third instar larva. (A) Larval epidermis shows strong fluorescent signal (green) at the apical epidermal surface (large arrows). This surface is covered by a cuticle (c), the outer regions of which autofluoresce (yellow). (B) Imaginal leg disc (ld) and eye disc (ed) from the same section as A. Signal follows the apical surface contours (as) of the folded disc epithelium. The basal surface (bs) is essentially unlabelled. (C,D) Optical sections of whole-mount preparations made by scanning confocal microscopy. (C) Wing disc from a 2 h prepupa with intense signal on the apical surface. (Horizontal bands of label represent accumulation of label in the grooves created by folding of the epithelium.) The basal surface is visible from faint background fluorescence. (D) A stage 10 or 11 h embryo (end of germ band extension). Signal is first seen in horizontal stripes but at a slightly later stage covers the entire apical cell surface. Only the posterior end of the embryo is shown. Primary sera were diluted 1:500 (A,B) and 1:1000 (C,D). No apically localized signal was seen with preimmune serum (1:500). No immunofluorescent signal was seen when either the primary or secondary antibody was absent.

appear to be located at the apical surface in both epidermal and disc epithelia. Thus, gp200, gp180 or both may be precursors to gp125. Alternatively, all three glycoproteins could be products of an insoluble precursor. We will refer to the precursor of gp125 as 'pre-gp125'. First we will discuss the evidence suggesting a role for the hormonally regulated proteolytic cleavage of pre-gp125 in imaginal disc morphogenesis. We will then speculate as to the possible nature of this molecule.

The importance of specific, localized proteolytic events in developmental processes has only recently been recognized (Chen and Chen, 1987; Saksela and Rifkin, 1988; Brenner *et al.* 1989). Notable examples in *Drosophila* are the involvement of serine proteases in the formation of the dorsal-ventral axis (DeLotto and Spierer, 1986; Chasan and Anderson, 1989). It has been known for some time that exogenous trypsin accelerates disc evagination (Poodry and Schniederman, 1971; Fekete *et al.* 1975; Nardi *et al.* 1987). Recently Pino-

Heiss and Schubiger (1989) demonstrated that discs synthesize and secrete several proteases including an ecdysone-dependent serine-protease suggesting that proteolysis is a normal part of evagination. Trypsin accelerated evagination is much more dramatic if the peripodial epithelium is disrupted or removed (Fekete *et al.* 1975; D. Fristrom, unpublished) consistent with the idea that trypsin acts primarily at the apical cell surface. Very little gp125 is generated and little evagination observed when intact discs (basal surface out) are incubated with trypsin. Thus, the apical location of pre-gp125 suggests that it may be a key target for exogenous trypsin. In contrast to trypsin, purified collagenase (trypsin-free) does not accelerate evagination, even though it strips the basal lamina from discs (D. Fristrom, in preparation) nor does it generate gp125. Morphogenesis can still be accelerated in collagenase-treated discs by subsequent addition of trypsin. Because the serine-protease inhibitor aprotinin prevents the appearance of gp125 in the presence of

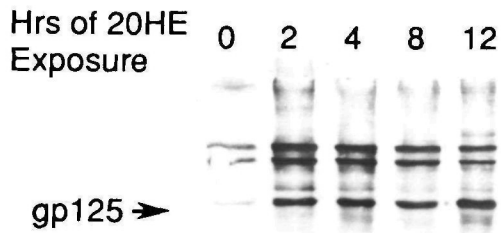


Fig. 8. Gp125 appears rapidly in response to hormone. SDS-soluble proteins from discs incubated with 20-HE for the times indicated were subjected to SDS-PAGE, transferred to nitrocellulose and detected with anti-gp125 antiserum.

hormone, pre-gp125 may be the substrate of an endogenous protease produced in response to 20HE. These results suggest, but do not prove, that proteolytic cleavage of pre-gp125 is required for disc morphogenesis. Such a proof awaits genetic analysis.

In addition to the production of gp125, several other lines of evidence suggest that substantial ecdysone-induced remodelling of the apical surface takes place during disc morphogenesis. The products of two genes expressed at the onset of disc morphogenesis, *IMP-E2* and *IMP-E3* (Natzle *et al.* 1987), have been localized to the apical surface (Paine-Saunders *et al.* 1990; Moore *et al.* 1990). Another apical extracellular matrix molecule, the assembly zone antigen (Wolfgang *et al.* 1987), shows rapid 20-HE-dependent accumulation at the disc apical surface. Rickoll and Galewsky (1987) have identified several antigens secreted by *Drosophila* S3 cells that also appear to be apically secreted in discs. Indeed, in insects, apical surface interactions with the extracellular milieu may be as important as basal interactions are for vertebrate morphogenesis.

It is clear that in discs, and probably also in the epidermis, gp125 is generated in response to hormone. Thus, it is worth speculating how proteolysis of pre-gp125 might function in *Drosophila* development. As a working hypothesis, we suggest that pre-gp125 may be a cell surface or transmembrane protein associated with the apical extracellular matrix (AECM). Staining patterns suggest that gp125 and its precursor(s) are closely associated with the apical cell surface rather than diffusely distributed in the apical extracellular space as is characteristic of AECM components (e.g. Fig. 15a, Wolfgang *et al.* 1987). The suggestion that pre-gp125 may be associated with the extracellular matrix is based on its insolubility in strong detergents. In the presence of Ca^{2+} this family of proteins is essentially insoluble even in Laemmli buffer. We therefore envision a Ca^{2+} -influenced association between this molecule and the

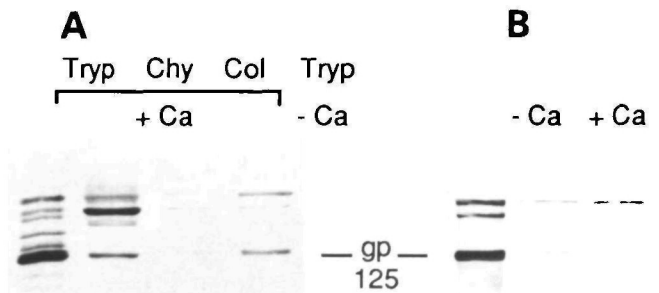


Fig. 9. The presence of Ca^{2+} effects both the generation of gp125 by trypsin proteolysis of discs and the detergent solubility of gp125. (A) Discs were homogenized and treated for 15 min with trypsin (Tryp), chymotrypsin (Chy) or collagenase (Col) in the presence of Ca^{2+} (+Ca). Trypsin generation of gp125 in the absence of Ca^{2+} (-Ca) is shown for a sample treated in parallel. Subsequent detergent extractions excluded divalent cations (TE buffers used). (B) Discs, incubated with 20-HE for 12 h, were sequentially extracted in detergents with buffers containing Ca^{2+} (+Ca) or excluding Ca^{2+} (-Ca). Gp125 and related antigens were detected by anti-gp125 antiserum on nitrocellulose after SDS-PAGE of the SDS-soluble fractions.

inherently insoluble extracellular matrix. The proteolytic cleavage that generates gp125 may disrupt this association, releasing constraints imposed by the AECM and thereby permitting morphogenetic cell movements. Such disruption may be important not only to imaginal discs but also to the epidermis. Pre-gp125 is present at the apical epidermal surface from mid embryogenesis, shortly before the deposition of the first cuticle. Cuticle is an elaborate apical extracellular structure. Attachment to the cuticle could be mediated by pre-gp125 and detachment (necessary for apolysis and molting) could require hormone-dependent proteolysis of pre-gp125.

If gp125 is indeed involved in the adhesion of the apical cell surface to the AECM, a number of important parallels can be drawn to other classes of cell adhesion molecules. The Ca^{2+} -dependent properties of the gp125 family of glycoproteins are reminiscent of those of cadherins, Ca^{2+} -dependent transmembrane glycoproteins involved in cell-cell adhesion (Takeichi, 1988). In the presence of Ca^{2+} , the cytoplasmic domain of epithelial cadherin (E-cad) appears to interact with the cortical cytoskeleton (Hirano *et al.* 1987). In this state, E-cadherin is insoluble in non-ionic detergent. In the absence of Ca^{2+} or when the cytoplasmic domain is removed, E-cad is free in the membrane and becomes extractable in non-ionic detergents (Nagafuchi and

Takeichi, 1988; 1989) providing a precedent for Ca^{2+} -dependent effects on solubility. Functionally however, pre-gp125 may be more akin to the integrins, highly conserved molecules involved in the adhesion of basal cell surfaces to basal ECM (Hynes, 1987). Both the cadherins and the integrins are associated with the cortical cytoskeleton and are thought to play an active role in signalling actin mediated events (Chen and Chen, 1987; BurrIDGE *et al.* 1988). With this in mind, it is intriguing to consider the possibility that proteolytic cleavage of pre-gp125 might play an active role in disc morphogenesis by initiating the contraction and/or protrusion of the apical actin network (Condic *et al.* 1990). The molecular and genetic characterization of the gp125 gene should allow us to test many aspects of this admittedly speculative hypothesis.

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